# TRANSFECTION OF CHIMERIC IMMUNOGLOBULIN GENES INTO LYMPHOID CELLS

Vernon T. Oi and L.A.Herzenberg [redacted]

The objective of this project is to transfect two chimeric immunoglobulin genes into a lymphoid tissue culture cell line capable of transcribing and translating these genes into proteins. The chimeric immunoglobulin genes will be constructed using standard recombinant DNA techniques and will consist of (1) a V-D-J gene segment coding for a dansyl hapten binding V-region and a Igh-b allotype constant region; and (2) a mouse V-D-J gene segment coding for a human cell surface antigen (e.g., Leu-2) and a human immunoglobulin constant region.

THE METHOD TO DELIVER DNA INTO THE CELL. There are currently five techniques being used to transfect DNA into eukaryotic cells. All five will be examined as possible means to introduce active immunoglobulin genes into lymphoid cells. The techniques include: (1) Ca-PO4 precipitation; (2) PEG 6000 fusion of lambda phage particles; (3) vesicle fusion; (4) protoplast fusion; and (5) microinjection.

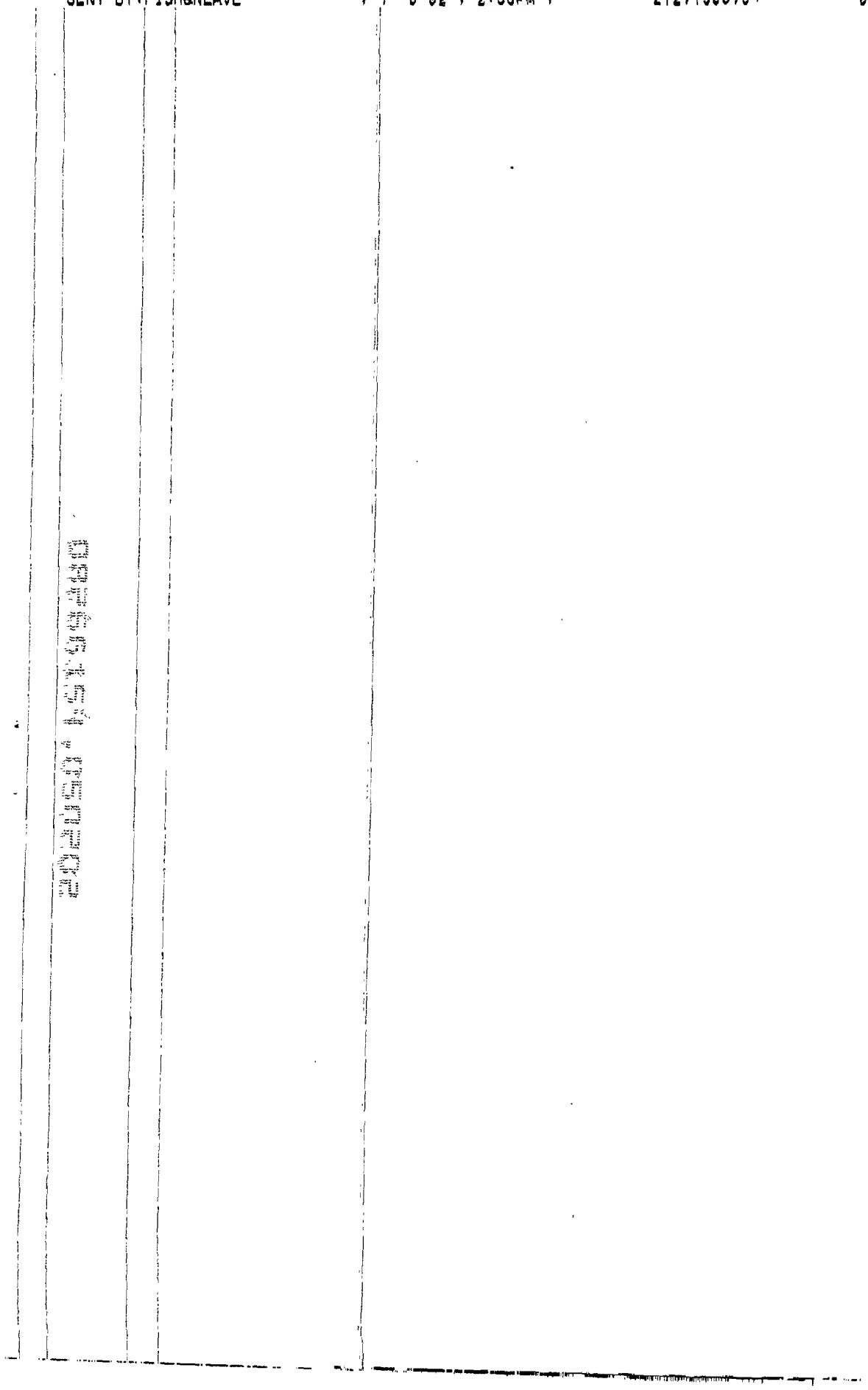
THE APPROPRIATE DELIVERY VECTOR. We have available to us suitable first generation SV40-pBR322 vectors to contain the recombinant immunoglobulin genes to be used in transfection experiments. Further development of these vectors also will be undertaken.

THE APPROPRIATE CELL HOST. Since the chimeric SV40-pBR322 vectors we are planning to use contain either the thymidine kinase or guanine phosphoribosyl transferase genes as selectable eukaryotic markers, we intend to develop lymphoid cell lines that lack these enzymes to use as transfectant recipients. These cell lines must have the potential to express immunoglobulin genes, but lack the ability to produce endogenous immunoglobulin products.

RECOMBINANT DNA. Standard recombinant DNA techniques will be used to isolate a DNS V-D-J gene segment from the genome of an existing hybridoma cell line producing anti-DNS antibodies. Igh-b constant region genes, as well as human constant region sequences will be isolated similarly. Chimeric recombinant V-D-J-Constant region sequences will be constructed from these newly isolated gene segments.

SELECTION OF TRANSFECTED CELL LINES EXPRESSING NOVEL IMMUNOGLOBULIN GENES. Should all of the above be accomplished, successfully transfected cell lines will be selected by enzyme markers (TK and GPT) and with the fluorescence-activated cell sorter using techniques and antibody reagents already developed.

Exhibit C



# APPLICATION FOR A RESEARCH OR CLINICAL INVESTIGATION GRANT



(Please read carefully the attached "Policies on Research and Clinical Investigation Grants" and the instructions on all pages of this form, before completing this application.)

TO: American Cancer Society, Inc.
777 Third Avenue
New York, New York 10017

Date [redacted]

Application is hereby made for a grant* in the amount of $[redacted]
for the period from [redacted] to [redacted] inclusive.

Title Expression of Transfected Mouse and Human-Mouse Hybrid Immunoglobul

FROM:

Sherie L. Morrison, Ph.D.
Name of Investigator

Sherie L. Morrison
Signature

Associate Professor of Microbiology
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Dr. Richard J. Sohn
Name of Official Authorized to Sign for Above Institution

Signature

Director of Grants and Contracts
Title

*It is understood that each applicant, by the act of applying for a grant, agrees, if the grant is made, to abide by the Society's POLICIES ON RESEARCH AND CLINICAL INVESTIGATION GRANTS.

Columbia University
Checks to be Made Payable to
Attn. Ms. Francy, Acting Controller
Box 6, Central Mail Room
Street and Number
New York, New York 10027
City, State, Zip Code

NO. 0058.02 – 1/82

# APPLICATION FOR A RESEARCH OR CLINICAL INVESTIGATION GRANT

## SUMMARY OF RESEARCH PROPOSED

Name and Official Title of Principal Investigator

Dr. Sherie L. Morrison, Associate Professor of Microbiology

Name and Address of Applicant Organization

Columbia University College of Physicians and Surgeons,
701 West 168th Street, New York, New York 10032

Title of Project

Expression of Transfected Mouse and Human-Mouse Hybrid Immunoglobulins

Use this space to summarize concisely your proposed research. Outline objectives and methods. Underscore the Key words (not to exceed 10) in your abstract.

Gene transfection has become an increasingly popular method of studying gene expression. We have recently developed methods of transfecting immunoglobulin genes into myeloma cell lines; these genes are efficiently expressed. The current experiments will define the regions of the mouse heavy and light chain genes which are required for efficient transfection and those required for high level immunoglobulin expression. Once these sequences are defined we will determine the influence of their position in the molecule on their function. We also will construct novel molecules and study their expression and function. In particular we will determine if hybrid molecules with the variable region from a mouse immunoglobulin (Ig) fused to the constant region of a human Ig molecule can be effectively produced and function. Secondly, we will examine the expression and function of molecules made from gene fragment. We will see if light chain dimers, one light chain of which has a heavy chain variable region can bind antigen. Such hybrid molecules have potential therapeutic value in treating human diseases such as cancer.

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2. Aim and Method of Study:

A. Specific Aims:

The aim of these studies is to produce novel immunoglobulin (Ig) molecules by using DNA mediated transformation of myeloma cells. The project will proceed in several steps.

a. Initially we will develop an optimum transfection system and define the regions of the mouse kappa light chain gene which are important for increased transformation frequencies. We will also investigate if other Ig genes contain sequences of similar function and attempt to define the mechanism leading to the increased transformation frequency.

b. Secondly, we will define the regions of both heavy and light chain genes which are required for efficient expression. Such a definition is required to permit the rational assembly of novel molecules which will be produced at high levels.

c. Thirdly, we will produce hybrid human-mouse Ig genes, test for their efficient synthesis in transfected cell lines, and assay the biologic activity of the novel molecules. We will also attempt to produce variant proteins of altered structure and function.

B. Methods

1. Immunoglobulin Genes To Be Used

In the initial studies we will use the heavy and light chain genes from the S107 myeloma. Genomic clones of both of these expressed genes have been obtained from Dr. Matthew Scharff and are available in the laboratory. Initial expression studies (see above) have focused on the S107A kappa chain gene. We will now also construct a vector containing the S107 heavy chain gene so that we can study its expression. To date three human heavy chain genes have been acquired. A VDJ segment and the $\gamma 1$ gene have been acquired from Dr. Honjo and a kappa chain gene from P. Leder. We will initially study the expression and function of mouse $V_L$-human $C_\kappa$ and mouse $V_H$-human $C_{\gamma 1}$ constructs.

2. Recipient Lymphoid Cell Lines

Our principal recipient cell line will be the mouse myeloma J558L. This produces a $\lambda$ light chain, no heavy chains and transfects very well. Because $\lambda$ and $\kappa$ are so different structurally we anticipate little competition between these molecules in assembly with heavy chain. However, if the $\lambda$ chain presents a problem we will isolate a non-producing variant of J558 using methodology which is routine in the laboratory.

3. Sequences Necessary for Efficient Transformation.

The S107A light chain gene is contained on a 7 Kb Bam Hl fragment as diagrammed in Figure 2. The deletions and partial molecules shown in Figure 2 have already been constructed and are being assayed for their transfection efficiency. Using the sites shown in the figure and others which we identify we will further assay the gene for transfection enhancement. The general protocol will be to subdivide the gene into fractions and assay each for its influence on transformation frequency. In particular we will put the Bam-Bgl or Bgl-Bgl pieces from the 7Kb L chain fragment into the Bam site of pSV2gpt and assay for transfection frequency. Other small fragments will be excised, blunt ended and Eco Rl or Bam Hl linkers put on. Bam Hl linkers have already been put on all the Hae III pieces from the L chain gene. Each fragment will be assayed for its enhancement of transformation; combinations of fragments will also be assayed to determine either synergistic or antagonistic interactions. Positive fragments will be subdivided into smaller pieces either by cutting with additional restriction enzymes or by cutting with progressive exonucleases such as Bal 31. The general objective will be to localize to as small a region as possible active sequences. The nucleic acid sequence of such regions will be determined and homologies between active regions sought.

Several possible mechanisms can be proposed to explain the increased transfection frequency; 1) replication of the plasmid as an episome; 2) increased expression of the selectible gene, in these experiments XGPT, or 3) increased integration into chromosomal DNA. We will try to distinguish among these possibilities.

Replication as an episome could be either transient during the early stages of the transfection or persistent. Transient replication increases the copy number of the plasmid within the cell and hence the probability of productive integration. To test for transient expression as an episome, 72 hours after transfection the Hirt supernatant (23) will be prepared from the transfected cell lines and the small molecular weight DNA examined by Southern (24, 25) blot after cleavage with the restriction endonuclease Mbo 1, and if available, Dpn I. Both Dpn I and Mbo 1 recognize the sequence GATC. If unmethylated this sequence is cut by Mbo I but not Dpn I; the sequence $G^{me}ATC$ is cut by Dpn I but not Mbo I. Since the dam methylase of E. coli introduces methyl groups on the $N^6$ position of adenine in the sequence GATC, while no eucaryotic enzymes do, it is possible to distinguish between DNA replicated in bacteria and that replicated in mammalian cells by the methylation pattern. To test for persistance as an episome, the Hirt supernatant will be isolated from stable transformants. Southern blot analysis will be done both on uncut DNA to test for the occurrence of DNA in the supercoil form and cut with restriction enzymes to assay for restriction fragments of the appropriate size. In addition, material from the Hirt supernatant will be used to transform bacteria. If replicating plasmids

are present they should be effective in transforming bacteria. If transformed bacteria are obtained, plasmid DNA will be isolated from them and the nature of the plasmid DNA determined following digestion with restriction endonucleases. In previous studies using these types of vectors, episomal replication has only been detected in Cos cells where T antigen is supplied in trans (26).

Analysis of stable transformants has already shown that the amount of gpt produced in those transfected with a pSV2gpt-S10721 is not consistently different from that produced in cells transformed using pSV2-gpt. However that does not exclude the possibility that increased transient expression of XGPT may lead to increased transformation. To test that possibility cytoplasmic extracts prepared from cells 48-72 hours after transfection will be assayed for their XGPT activity (9 and appended reprint). In the vector which we have routinely used for transfection, the SV40 early promoter has been used to drive the bacterial XGPT gene. It is also possible to use the promoter from the Herpes thymidine kinase gene (appended manuscript) to drive the XGPT gene. We will assess if sequences effective in enhancing transfection by vectors using the SV40 promoter are effective with the TK promoter and if these sequences lead to increased transient expression of sequences off the TK promoter.

It is also possible that increased transformation results from an increased frequency of vector integration into chromosomal DNA. It is difficult to directly test this hypothesis. However we will do Southern blot analysis following cleavage with restriction endonucleases with 6 base recognition sequences of DNA isolated from transformants obtained using vectors either with or lacking the enhancing sequences. This analysis will give us an estimate of the number of sites of integration per transformant. If the same size restriction fragments are found in independent transformants it will suggest a common site of integration. To confirm this it would be necessary to clone the integrated genes and directly analyze the flanking sequences. Methods to produce genomic libraries using lambda phages are available in the laboratory.

4. <u>Identification of Genetic Sequences Necessary for High Level Immunoglobulin Expression.</u>

Preliminary experiments have demonstrated that it is possible to introduce a rearranged mouse kappa light chain gene back into a mouse myeloma cell by DNA mediated transformation; the reintroduced light chain can be expressed within the myeloma cell to levels approaching that of the endogeneous myeloma light chain. Deletion analysis has also suggested that sequences within the IVS are required for efficient Ig expression. By cutting with Hind III we can now mix and match the 5' and 3' deletions. We will do these experiments to precisely define the extent of the region necessary for expression. Once we have appropriately located the sequences, we will make additional Bal 31 deletions to try and locate the sequences to within one or several

nucleotides. The end points of the deletion will be sequenced and compared to the published sequence of the IVS (14) to accurately position them.

Once the IVS necessary for high level Ig production has been accurately identified we will do further analysis of the effects of this sequence and the structural requirements for its function. We will determine if there is a position effect on Ig production, that is, must the sequance always be at the same position and in the same orientation in the Ig gene to exert its enhancing effect. The SV40 enhancers provide an example of an enhancer that functions in various positions and orientations. The Ig sequences which failitate Ig production will be placed both 5' and 3' of their normal positions in the Ig gene and elsewhere in the expression vector in either orientation and the level of Ig expression assayed. Linkers will be put on the active fragment. By using linkers, we will invert the sequence in its normal site, and also duplicate it in both its normal and inverted orientation. Random small insertions (21) will also be put into the active sequence to define its structural requirements for function. We will make constructions with IVS consisting only of the required sequence and enough information to preserve the 5' and 3' splice junctions. In addition we will determine if the Ig sequences increase the expression of genes being synthesized off non-Ig promoters. Vectors exist with the bacterial XGPT gene being expressed using either the SV40 or the Herpes thymidine kinase (TK) promoter. The Ig sequences will be placed at various positions relative to the SV40 and TK promoters and the synthesis of XGPT assayed both in transient expression experiments and in stable transformants.

We will also test for the influence on expression of sequences 3' to the coding region. We have available a kappa cDNA clone with R1 ends. We will convert these R1 ends to BAM ends by blunt ending with S1 or T4 polymerase and adding BAM linkers. We will then exchange the 3' Hpa 1-Bam fragment from the cDNA for the same fragment from the pSV2-S107-21 vector. The resulting vector will lack sequences 3' to the mRNA. If this light chain is efficiently expressed we will do Bal 31 digestion before putting on the Bam linkers. Exchange of the Hpa-Bam fragments after Bal 31 digestion will delineate how much of the 3' sequence is required and if it is necessary to have a poly A addition site. We can add back a poly A site from SV40 to provide a new poly A site at a different position.

The sequences 5' to the gene necessary for expression will also be determined. Preliminary construction will be done by cutting with R1 + Pvu II and R1 + partial Xba, putting on R1 linkers, reclosing and assaying. Bal 31 digestion can be done before putting on the linkers to more accurately define the required sequences. The present experiments will be designed merely to identify the extant of the necessary sequences. Fine structure mapping of the promoter sequences by such methods as <u>in vitro</u> mutagenesis and "linker scanning" (21) are beyond the scope of the present proposal.

The experiments detailed about all relate to expression of the kappa chain gene. A similar series of experiments will be done to identify IVS, 5' and 3' sequences necessary for expression of the S107 H chain gene. For H chains we will also determine if the synthesis of a light chain, either specific or non-specific, is required for or facilitates expression.

To assay for the synthesis of the transfected gene product cells will be labeled with $^{14}$C-valine, threonine, and leucine, cytoplasmic extracts made (27) and the Ig immunoprecipitated. Specific immunoprecipitable chains will be identified using SDS gels. We have found that the S107 kappa chain can easily be separated from the J558 lambda chain using SDS-$PO_4^-$ gels (unpublished results). In selected experiments 2-D gels also will be used to identify the products of transfected genes (7).

The amount of the transfected product synthesized will be quantitated in two ways. Firstly, the ratio of the amount of synthesis of the endogeneous immunoglobulin light chain to the transfected light chain will be determined by scanning the autoradiograms of SDS gels of immunoprecipitates from transfected cells. If labeling is done for a short period of time so that neither chain is secreted or significantly degraded this method gives a good estimate of the relative rates of synthesis. To quantitate the synthesis as a percentage of the total protein synthesis, cells will be labeled for short periods of time (3-5 minutes) with $^{14}$C-amino acids, the total amount of TCA precipitable material synthesized determined, and the amount of TCA precipitable material which is immunoprecipitated determined. Pulse chase experiments will be used to determine the rate of degradation of the immunoglobulin. Long term (3-24 hours) labeling with $^{14}$C-valine, threonine, and leucine, immunoprecipitation and SDS gel analysis of the secreted product (with and without reduction) will determine what product is secreted and whether it is assembled.

Northern blot analysis and hybridization with Ig specific $^{32}$P-labeled probes will be used to determine the approximate size and heterogeneity of any Ig specific transcripts in the cell lines. Formaldehyde gels and the blotting procedure of Thomas (28) is used routinely. In the cases where the recipient cell line synthesizes an immunoglobulin with the same constant region as the transfected gene, variable region probes will be used.

The 5' and 3' end of the cytoplasmic transcripts and points of splicing of the IVS will be mapped using the S1 nuclease resistance method of Berk and Sharp (29). In the case of the S107A gene the plasmid will be labeled at the Hpa I site in the constant region using T4 polymerase and the 1.5 Kb Hpa I to BAM HI fragment used to identify the 3' end of the transcript. Label of the Hpa I site with kinase will be used to position the 3' end of the IVS and label of the Kpn site within V with T4 polymerase will be used to locate the 5' side of the IVS.

Because there is an IVS between the leader sequence and $V_L$ and no known unique restriction site in the leader sequence, templates synthesized in M13 will be used to map the 5' end of the transcripts. Hind III linkers have already been attached to the Hae III fragment which contains the region 5' to the light chain gene and the 5' end of the variable region and should contain the light chain promoter region. This fragment will be cloned into M13, and used to synthesize message complementary probe for S1 mapping experiments. If some transcripts originate 5' of this fragment, a larger fragment will be cloned into M13. S1 analysis will be done on RNA isolated from both the transient expression experiment and from stable transformants. We have already used S1 analysis to demonstrate that the 3' ends of the mRNA from transient expression and stable transformants with many of the vectors are identical.

The Northern blot and S1 analysis will yield information about the structure of steady-state cytoplasmic mRNA. To gain some information about nuclear RNA, it will be isolated from selected transformants and the size of the nuclear transcripts determined by Northern blot analysis. Initial blotting will be done with probes which contain the entire Ig gene. Region specific probes will be used to both elucidate the pattern of processing and to identify abnormal transcripts. A necessary control for these experiments will be a careful analysis of the nuclear RNA of the recipient cell lines to eliminate the possibility that they contain aberrant transcripts of Ig genes.

5. Expression and Function of Novel Immunoglobulin Molecules

Once we have a clear idea of the sequences necessary for efficient Ig production we will begin to construct novel Ig molecules and will study their expression and function. Combinations which we will produce include:

a. [S107 kappa] + [S107 alpha]
b. [$V_H$ S107 + γ1 human] + [S107 kappa]
c. [$V_L$ S107 + κ human] + [S107 alpha]
d. [$V_H$ S107 + γ1 human] + [$V_L$ S107 + κ human]

In these constructions both the H and L chain will be covalently linked into the expression vector to increase the probability of their cotransformation and expression.

Combination a will demonstrate that it is feasible to establish an antigen binding cell line by gene transfection. Combinations b and c will demonstrate whether it is possible to get expression of hybrid molecules, and if it is possible to assemble molecules, one constant region of which is of murine origin, the other of which is human. Combination d will demonstrate if it is possible to express a molecule with the specificity of murine origin, but the constant region and effector functions of human origin.

If we achieve efficient expression using the entire gene we will make and analyze a series using only gene fragments. Among the combinations which we plan are:

a. [$V_L$ S107 + $C_\kappa$ human] + [$V_H$ S107 + $\gamma$1 human with $CH_1$ deletion] / $CH_2$ deletion] / $CH_3$ deletion] / $CH_2$ + $CH_3$ deletion] / $CH_1$ + $CH_2$ deletion] / $CH_1$ + $CH_3$ deletion]

b. [$V_L$ S107 + $C_\kappa$ mouse] + [$V_H$ S107 + $C_\kappa$ mouse]

c. [$V_L$ S107 + $C_\kappa$ human] + [$V_H$ S107 + $C_\kappa$ human]

All transformants will be assayed, using the methods detailed above for the synthesis, assembly and secretion of Ig molecules. Transcripts will be analyzed both for their fidelity and quantity.

One of the reasons for using the S107 $V_H$ and $V_L$ is that they come from a molecule of known antigen specificity, an anti-phosphorylcholine antibody. Recombinant molecules will therefore be assayed for their ability to bind phosphorycholine (PC). This can efficiently be done by labeling the proteins by growing the cells in $^{14}$C-VTL and then testing for binding to PC-Sepharose. The proteins binding will be analyzed on SDS gels following elution. Human $\gamma$1 fixes complement. If recombinant molecules bind antigen, their ability to fix complement will be tested. Resistance to serum protein proteases will be tested by incubating biosynthetically labeled proteins in serum at 37°C for varying lengths of time, and then analyzing the amount of Ig which can be immunoprecipitated. Immunoprecipitated material will be run on SDS gels to determine its size. Serum half-life will be tested by injecting biosynthetically labeled proteins into mice and following their serum decay. It would be desirable to assess these parameters in humans, but such experiments are beyond the scope of this grant.

Extensive death of 9D36 and A20 cells
Repeat
Centrifuge PEG – 0.5 min
After adding DMEM centrifuge 3 min
Improvement but still extensive

For stable transfectants

1 ml J558L cells - x 2
1 ml VHHUG2 x 2
1 ml CK7 x 2
1 ml HUK x 2
0.5 ml HUK + 0.5 ml HUF x 2
0.5 ml CK + 0.5 ml 5107.16 x 2

plate into microwell dishes

Yield - least 6 stable cells subclones per well
95 % per year

# 4. TRANSFECTION BY PROTOPLAST FUSION

Reagents:

| | |
|---|---|
| LB + ampicillin (must be less than 1 month old) | |
| LB + 1% glucose | |
| LB + Glu + chloramphenicol (1.25 mg/ml) (10X) | |
| 20% sucrose in 50 mM Tris pH 8.0 | AUTOCLAVED |
| 250 mM Tris pH 8.0 | USE AT 4°C |
| 50 mM Tris pH 8.0 | |
| 250 mM EDTA pH 8.0 | |
| 35% PEG 1500, 100 mM Tris pH 8.0 in DME | FILTER STERILIZED |
| OR | USE AT |
| 41.7% PEG 1500, 12.5% DMSO, 100 mM Tris pH 8.0 in DME and | 37°C |
| 50% PEG 1500, 100 mM Tris pH 8.0 in DME | USE AT 37°C |
| DME + 10% ultra pure sucrose + 10 mM $MgCl_2$ | |
| pH the DME based solution with $CO_2$ | |
| NOTE: The pH of these solutions will change with storage. Check and adjust the pH before use. | |
| DME pH 8.0 | USE AT 37°C |
| Growth media + 1% gentamycin (100 ug/ml final concentration) + 1% nystatin | |
| 5 mg/ml lysozyme in 250 mM Tris, pH 8.0. Make up fresh. | MAKEUP FRESH FILTER STERILIZE USE AT 4°C |

Equipment:

37°C water bath
Ice buckets
250 ml sterile centrifuge bottles
Pasteur pipets
IEC centrifuge at room temperature
Sorvall centrifuge
50 ml tubes

Reference:

Oi, V. T., Morrison, S. L., Herzenberg, L. A., and Berg, P.: Immunoglobulin expression in transformed lymphoid cells. Proc. Natl. Acad. Sci. U.S.A. 80:825, 1983.

PROCEDURE:

2 DAYS BEFORE Start 1-2 ml overnight in LB + amp.

1 DAY BEFORE Inoculate 100 mls of LB + 1% glucose with 1.0 ml of overnight culture. Shake in the warm room on the wrist action (preferably) or at 200 rpm on the platform shaker.

Grow until $O.D._{600}$ = 0.5-0.7. Begin checking O.D. after 2 hrs. When O.D. reaches 0.5, add 10 mls of 10X chloramphenicol which is stored in the door of the refrigerator next to the incubator. Return to shaker and let go overnight.

FUSION DAY Pellet bacteria in sterile 250 ml centrifuge bottles at 6000 rpm for 5 minutes in the Sorval at 4°C; reserve 1.0 ml for taking the O.D.

Reserve 1.5 ml for a mini-prep.

Meanwhile:

Chill necessary solutions on ice
Warm necessary solutions to 37°C
Take O.D. of bacteria
Prepare and filter sterilize lysozyme

PROTOPLAST PREPARATION:

Take bacteria out of the centrifuge and put on ice. Transport to the hood. Tilt the bottles with the pellet facing toward you and aspirate off all the supernatant.

All remaining manipulations are done on ice in the hood. Caps may be left off the bottles until bottles are removed from the hood.

Add 1.0 ml of cold 20% sucrose in 50 mM Tris pH 8.0 / 0.1 O.D. unit (e.g., 5.0 ml/0.5 O.D.). Gently pipet up and down until well mixed. Discard any excess volume over 5.0 ml. If O.D. is less than 0.5 do no adjust the volume down. Treat as though the O.D. = 0.5 and use more protoplasts for fusing.

Add 1.0 ml of lysozyme solution slowly and swirl gently. Incubate on ice for 5 minutes.

Add 2.0 ml of cold 250 mM EDTA pH 8.0 slowly and swirl gently. Incubate for 5 minutes on ice.

Add 2.0 ml of cold 50 mM Tris pH 8.0. Swirl gently, cap bottles and quickly incubate at 37°C for exactly 8 minutes and 30 seconds.

You can use a "temp block" to weigh down bottles that float in 37°C water bath.

Return bottles to hood. Remove caps. Add 5.0 ml of DME + sucrose + $MgCl_2$ to each bottle while gently swirling. Add another 5.0 ml to each bottle while swirling. Add 10.0 ml to each bottle while swirling. Add 20 ml to each bottle while swirling for a total of 40 ml. It is important that the medium is warmed to 37°C.

Allow protoplasts to stand at room temperature for at least 30 minutes before use.

Empty suction bottles.

TUBE FUSIONS:

Add 2-5 x $10^6$ cells per tube.

Spin cells down and aspirate off media.

Wash cells 1 time in DME. Aspirate off media.

Add 5.0 ml of protoplasts. Pipet up and down to mix with cells.

Spin tube 5 minutes at 1,600 rpm in the old IEC at room temperature.

Aspirate medium; resuspend pellet by hitting tube with finger.

Add 1.0 ml 35% PEG in 100 mM Tris at 37°C. Centrifuge for 1.5 minutes at 1,600 rpm with the brake on. Disrupt the pellet with 12 ml of warm DME. Spin 5 minutes at 1,600 rpm. Aspirate supernatant and add 12 ml of growth media. Mix well. Plate two drops per well in a 96 well plate or put the 12 mls in a 25 ml petri dish. Place in incubator as soon as possible.

OR

Add 0.5 ml of PEG-DMSO at 37°C; shake gently 1.0 minute. Add 0.5 ml of 50% PEG; shake 2 minutes. Disrupt the pellet with 10 ml of warm DME. Spin 5 minutes at 1600 rpm. Aspirate supernatant and add 12 ml of growth media. Mix well. Plate two drops per well in a 96 well plate or put the 12 mls in a 25 ml petri dish.

Two days later add 2 drops per well of selective media.

Four days after adding selective media, aspirate off half of the media and add fresh selective media.

PLATE FUSIONS:

Use 6 well plates.

Each fusion point is divided between two wells.

Add 1.5 x $10^6$ cells per well. Add 2.5 ml of protoplast per well.

Spin plates in the old IEC for 5 minutes at 1,600 rpm with the brake off. Hang plates over the outside edge of the centrifuge holders.

Place plates on a slant on the front edge of the hood and with a pasteur pipet at the surface of the media, carefully aspirate off the media.

Place the plates flat. Add 1.0 ml of 37°C 35% PEG. Spin 1.5 minutes at 1,600 rpm with the brake on. Disrupt pellet with 6.0 ml of warm DME.

Spin 5 minutes with the brake on at 1,500 rpm. Aspirate media.

Resuspend each well in 6.0 ml growth media and mix the two wells together.

Plate in a 96 well plate at two drops per well. Two days later add 2 drops per well of selective media. Four days later aspirate off half of the media and add fresh selective media.

HOW TO PREPARE PEG SOLUTIONS:

BDH PEG 1500 - ordered from Gallard-Schlesinger.

Aliquoted stock is stored in a box over Letitia's desk.

Melt PEG at 65°C. Cool to around 42-48°C. Add 42°C DME.

Add 1 M Tris pH 8.0 to a final concentration of 100 mM. Use unautoclaved Tris.

If making PEG-DMSO, use the Fisher DMSO that has been sterile filtered and stored in the freezer.

[DATE REDACTED]

EFFICIENCY LINE- 22-203

Take 1.4 ml of material bound to PC column and eluted with PC
add 50 µl of Staph either uncoated or coated as prepared on 5/4
incubate together
wash 4x in Tris-saline
dissolve in 60 µl of SDS Simple buffer - count 5 µl
also count 200 µl of supernatant

| | | | | | | |
|---|---|---|---|---|---|---|
| Staph 120 139 R4 154 | 2106 | 1.00 | 113.0000 | 192.0000 | .588500 | ppts |
| | 2107 | 1.00 | 236.0000 | 447.0000 | .514700 | |
| | 2108 | 1.00 | 113.0000 | 287.0000 | .380400 | |
| | 2109 | 1.00 | 112.0000 | 289.0000 | .374800 | |
| | 2110 | 1.00 | 184.0000 | 256.0000 | .430500 | |
| | 2111 | 1.00 | 5.0000 | 2.0000 | 2.500000 | |
| | 2116 | 1.00 | 127.0000 | 181.0000 | .701600 | supernatant |
| | 2117 | 1.00 | 95.0000 | 226.0000 | .420300 | |
| | 2118 | 1.00 | 126.0000 | 244.0000 | .516500 | |
| | 2119 | 1.00 | 106.0000 | 260.0000 | .415300 | |
| | 2120 | 1.00 | 87.0000 | 206.0000 | .466200 | |
| | 2121 | 1.00 | | | | |
| | 2126 | 1.00 | 221.0000 | 697.0000 | .369000 | — 1 µl / 50 µl column flow thru |
| | 2128 | .64 | 1.600 | 1.600 | 1.000000 | |

Staph #

| | Staph | 120 | 139 | R4 | 154 |
|---|---|---|---|---|---|
| total ppt | 2400 | 3000 7800 | 3600 | 3600 | 3600 |
| Total remaining | 360 | 1562 | 1760 | 1820 | 1400 |
| cts ppt from flow-through | | | | | 45,000 |

Exhibit B

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# THE USE OF MUTANT MYELOMA CELLS TO EXPLORE THE PRODUCTION OF IMMUNOGLOBULINS

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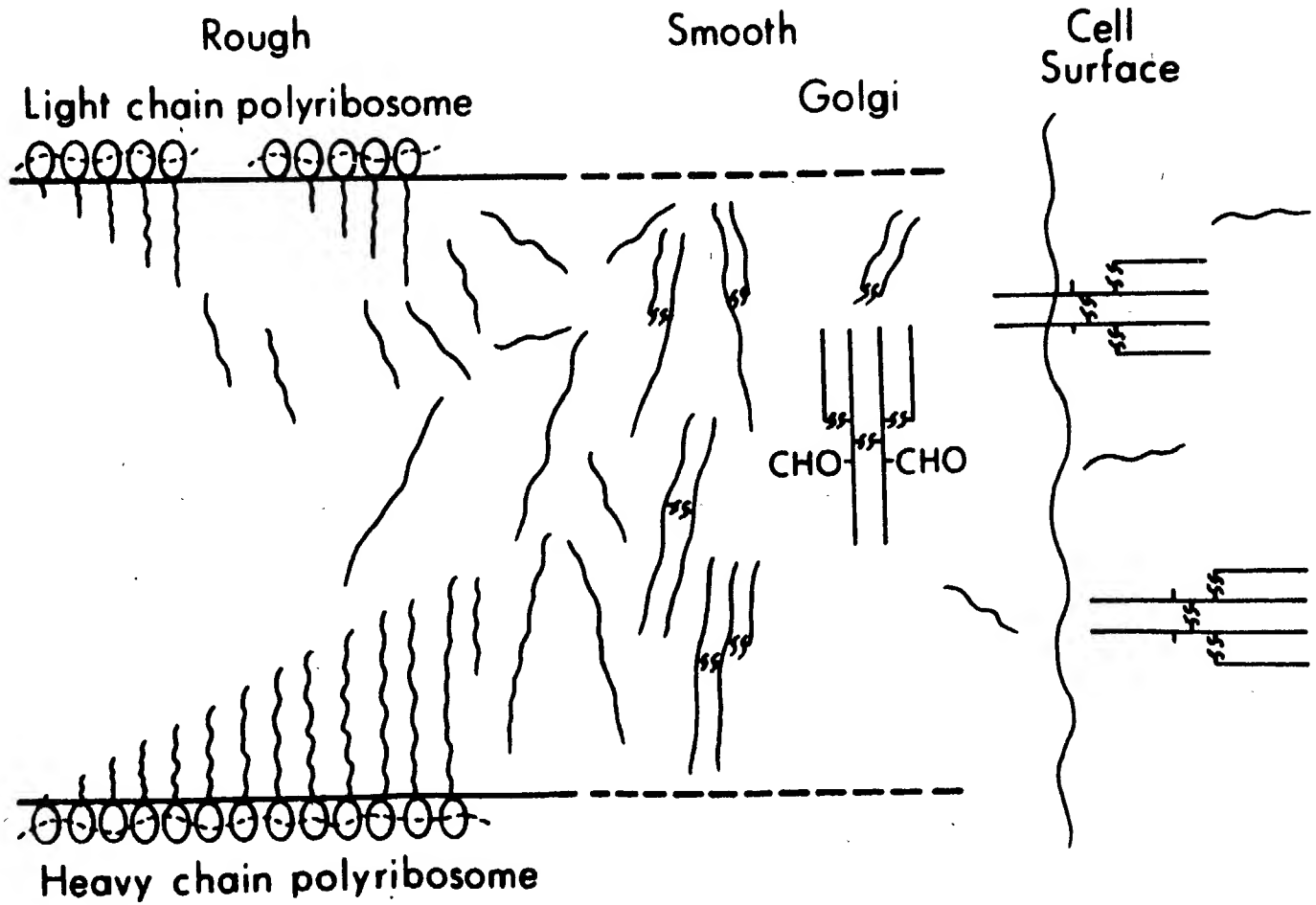
Abstract: A method has been developed for identifying and quantifying mouse myeloma cells which have undergone mutations in immunoglobulin production. Such variants arise at a very high rate both spontaneously and with mutagenesis. Mutagenesis results in primary sequence variants which are blocked in a variety of steps in the synthesis, assembly, glycosylation, and secretion of H chains. These mutants can be used to investigate the production and genetic control of immunoglobulins.

## INTRODUCTION

Studies carried out in a number of laboratories have provided a good general description of the synthesis, assembly, and secretion of mouse immunoglobulin (1,2,3). These studies have been done both with mouse myeloma and normal lymphoid cells. The molecular events involved in the production of IgG immunoglobulins are presented schematically in Figure 1. The immunoglobulin messenger is transcribed and processed in the nucleus, and transported into the cytoplasm where it becomes associated with membrane bound ribosomes to form the rough endoplasmic reticulum. There it is translated into heavy (H) and light (L) chains (1,2), which in mouse myeloma cells together represent 20 to 40% of the newly synthesized protein (4). These H and L chains are released into the cisternae of the endoplasmic reticulum and assembled through a variety of covalently linked intermediates into the fully assembled $H_2L_2$ IgG molecule. The H chains, and in some cases the L chains, are glycosylated in both the rough and smooth endoplasmic reticulum (5,6,7). Most of the immunoglobulin molecules are secreted relatively rapidly, but a small percentage is inserted into the plasma membrane and remains there for many hours (5,8).

Figure 1


Rough
Smooth
Cell
Surface
Light chain polyribosome
Golgi
CHO
CHO
Heavy chain polyribosome

The events depicted in Figure 1 raise a number of interesting questions. Since the genes coding for H and L chains are unlinked (9), are approximately equimolar amounts of the two polypeptide chains synthesized? If so, what is the mechanism of coordinated control? We would also like to know what determines the kinetics, pathways, and completeness of assembly, whether the carbohydrate plays any role in assembly or secretion, and why some molecules remain associated with plasma membrane while others are secreted? Most importantly, is there some way that we can use the highly differentiated and malignant myeloma cells which synthesize large quantities of a homogeneous immunoglobulin to learn something about the unique aspects of the genetic control of immunoglobulin production. For example, can we learn how two genes code for one polypeptide chain, why only one of the two alleles is expressed, or if there is any somatic contribution to the generation of antibody diversity?

Attempts to answer these and other questions and to understand the detailed mechanism responsible for each of the steps in production of immunoglobulin would be greatly facilitated if a series of mutants blocked at each step in the process could be isolated and characterized. Additionally, myeloma cells with mutations in the H or L chain genes would be very useful in studying the somatic cell genetics of animal cells since large amounts of the mutant gene products could be purified and characterized chemically.

## THE ISOLATION OF VARIANTS

Mouse myeloma cells growing in continuous culture are cloned in soft agar. In order to obtain high cloning efficiency, nearly confluent monolayers of primary, secondary, or tertiary rat embryo cells are used as feeders. These are overlayed with agarose in growth medium after which a single cell suspension of myeloma cells in very soft agar is layered on top of the base layer (Figure 2). With most cell lines

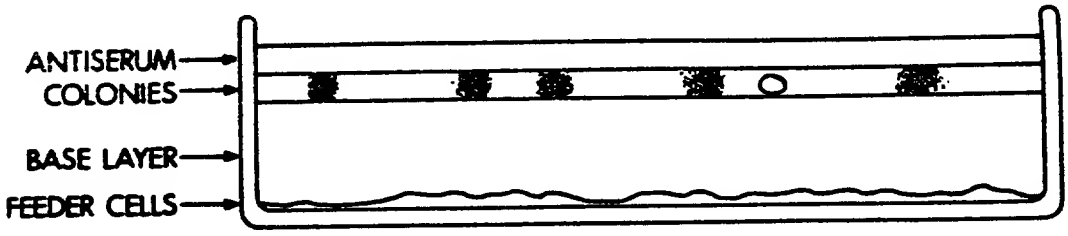



Figure 2

50-100% of the myeloma cells form clones. When the growing clones contain 4-16 cells, a third layer of agarose containing high titre rabbit antiserum against mouse heavy chains is placed over the clones (Figure 2). Two to three days later the plates are screened under medium power with an inverted microscope. Cells producing normal heavy chain containing molecules secrete them into the surrounding agar. The antibody diffuses down into the agar to form a visible antigen-antibody precipitate which surrounds and obscures the clone (10). All colonies which are not surrounded by precipitate are presumptive variants and can be recovered from the agar, grown up to mass culture, and characterized biochemically and serologically.

Such presumptive H chain variants could have three different sets of defects: 1) loss of ability to synthesize H chains; 2) defects in assembly or secretion; and 3) the synthesis and secretion of defective heavy chains which no longer react with the antiserum used. If antiserum against L chains is used, then cells with similar defects in L chain production can be identified.

## TYPES OF SPONTANEOUS VARIANTS AND THE FREQUENCY WITH WHICH THEY OCCUR

When freshly isolated clones from the MPC-11 ($IgG_{2b}$), P3 ($IgG_1$), MOPC-31 ($IgG_1$) or C1 ($IgG_{2a}$) cell lines were examined for variants with antibody against H chains, 0.5 - 1% of the clones were unstained. Because of the unexpectedly high frequency of variants, fluctuation analysis (11) was carried out to determine if the variants arose spontaneously and the exact rate at which they were being generated. All three lines lost the ability to produce H chains at the rates of $1\text{-}2 \times 10^{-3}$/cell/generation, or one in every thousand cell divisions resulted in a "mutation" (10). This enormous instability of immunoglobulin production is also found in IgA and IgM producing mouse myeloma cell lines and seems to be peculiar to immunoglobulin production. The same cells became resistant to four different drugs at a frequency of $10^{-6} - 10^{-7}$/cell/generation, which is many orders of magnitude lower than the spontaneous instability of immunoglobulin production (12).

Over a hundred MPC 11 and P3 variants which had spontaneously lost the ability to secrete H chains have been examined by incubating with radioactive amino acids for intracellular H chains. Short labelling times were used to rule

out synthesis and subsequent degradation. Antiserum raised against denatured H chains was used to increase the chance of precipitating fragments or molecules which had lost many of the normal antigenic determinants. No detectable H chains were found. We concluded that such spontaneously occurring variants were synthesizing less than 1/50 - 1/100th of the amount of complete H chains produced by the parental H plus L chain producing cells (10,13). Eleven P3 variants synthesizing only H chains and over thirty P3 and MPC 11 variants which no longer secreted either H or L chains were also studied and the missing chains were not detected intracellularly.

One of the non-producing variants has been examined in detail for L chain messenger RNA translatable in heterologous cell free systems or hybridizable to cDNA (14). No messenger for complete L chains was detected. Revertants have also been sought and not found. Without revertants or mutant gene products it has not been possible to determine the mechanism responsible for these spontaneously occurring variants.

## VARIANTS ARISING THROUGH MUTAGENESIS

In an attempt to find variants making defective gene products which could be characterized chemically, we have examined the effect of a number of mutagenizing agents on the types and frequency of variants. Ethylmethane sulfonate did not increase the frequency of variants. Nitrosoguanidine caused a 2-3 fold increase in variants. However, ICR 191, which is an acridine half mustard that causes frame shift mutations in microorganisms, caused a very significant increase in variants (12) (Table 1). Melphalan, a phenylalanine mustard used in the treatment of human multiple myeloma, is also an effective mutagen (15) (Table 1). Most of the mutagen induced variants resembled those that occur spontaneously in that they have lost the ability to synthesize one or both of the immunoglobulin polypeptide chains. However, 30-40% of the unstained clones continued to synthesize H chains which were chemically and serologically different from those produced by the parental cells. Twenty such mutants with changes in the primary sequence of their heavy chains have been studied in more detail (16,17). The general serological and chemical characteristics of three representative primary sequence mutants are summarized in Table 2.

TABLE I

Effect of mutagens on the incidence of variants

| | Mutagen (μg/ml) | % Cell survival | Incidence of variants | % Variant | p |
|---|---|---|---|---|---|
| ICR 191 | 0 | 100 | 18/2104 | 0.86 | |
| | 1 | 60 | 56/3635 | 1.54 | 0.016 |
| | 2 | 25 | 110/3404 | 3.24 | <0.001 |
| | 4 | <1 | 15/229 | 6.55 | <0.001 |
| Melphalan | 0 | 100 | 17/3777 | 0.45 | |
| | .2 | 32 | 31/2336 | 1.33 | <0.001 |
| | .4 | 28 | 100/5926 | 1.65 | <0.001 |
| | .6 | 16 | 55/2961 | 1.86 | <0.001 |
| | .8 | 9 | 31/1298 | 2.39 | <0.001 |

Briefly summarized, the variants fall into two groups. The first set synthesize short H chains ranging in different variants from 39,000 to 50,000 daltons compared to 55,000 daltons for the parent. Those presented in Table 2 all have the same variable region antigenic determinants (idiotype) a the parent but others not shown are either less reactive or not reactive with idiotypic antibody (18). When examined with antibody against denatured H chains, all of the short chain variants lack some of the antigenic determinants found on the parent and this is confirmed by their lack of reactivity both with antibody against the Fc region of the parent and with antibody against the $IgG_{2b}$ subclass specific determinants of the parent (Table 2)(16). Comparison of tryptic-chymotryptic digests of the variant and parental H chains reveals that most of the 35 peptide peaks visualized are identical but that 4-6 of the peaks present in the parent are missing from the variant and a few new peaks are detected in the variant (16). When a 39,000 dalton H chain is compared to a 50,000 one, they both lack some of the same peaks but fewer are missing from the larger chain. Furthermore, we have observed the conversion of a variant synthesizing a 50,000 dalton H chain to one synthesizing a 39,000 dalton H chain. Dr. Birshtein is currently carrying out detailed structural studies on these variants and one possibl interpretation is that these short chain variants are missing the C-terminal portion of their H chains. A similar type of variant has been reported to have arisen spontaneously in the P3 cell line (20).

TABLE II

| Mutagen | Molecular weight of H chain | Cytoplasmic lysates: denatured H chain | Cytoplasmic lysates: idiotype | Cytoplasmic lysates: Fc | Cytoplasmic lysates: $IgG_{2b}$ | Cytoplasmic lysates: $IgG_{2a}$ | Secretion: denatured H chain | Tryptic chymotryptic peaks in common with parent |
|---|---|---|---|---|---|---|---|---|
| 0 | 55,000 | + | + | + | + | - | + | |
| Melphalan | 39,000-44,000 | +S | + | - | - | - | + | 27/35 |
| ICR | 50,000 | +S | ND | - | - | - | - | 26/30 |
| Melphalan | 55,000 | +S | + | +S | - | + | + | 18/34 |
| ICR | 75,000 | +S | + | +S | - | + | - | 18/34 |

Cells were concentrated by centrifugation, lysed with Nonidet P-40 and the cell lysates examined with the indicated antisera by double diffusion in agar. "S" indicated a spur with the parent containing antigenic determinants not found in the variant. Idiotypes were examined by R. Lieberman and M. Potter of the NIH using hemagglutination inhibition. Peptide analysis was carried out by mixing $^{3}H$ amino acid labelled parental H chains with $^{14}C$ amino acid labelled variant H chains, digesting and then chromatographing the digest as described previously (16).

The second set of variants synthesize normal size or large (75,000 dalton) heavy chains. Again the ones described in Table 2 have the same idiotypic determinants as the parent but others are less reactive (18). Like the short chain mutants, these lack antigenic determinants thought to be located in the C-terminal portion of the H chain. However, the distinguishing characteristics of these mutants is that they have not only lost the $IgG_{2b}$ subclass specific determinant found in the parent but have also acquired antigenic determinants distinctive for the $IgG_{2a}$ constant region gene which was not expressed in the parent. Instead of lacking only a few of the tryptic-chymotryptic peptides of the parent, less than half of the parental peptides are found in these "2a positive" H chains. Amongst the many new peptides seen, at least five are present in another $IgG_{2a}$ but not in other $IgG_{2b}$ heavy chains (Table 2)(17). Different 2a positive mutant H chains all differ from each other by peptide analysis. The L chains of all of the variants so far studied are identical with parent by peptide maps.

The phenotypes of all of these primary sequence variants as well as of all of the other MPC 11 and P3 variants characterized so far are presented in Table 3. These variants can be used to begin to answer some of the general questions raised earlier.

## COORDINATION OF H AND L CHAIN SYNTHESIS

As already noted, H and L chains are synthesized in large amounts by the mouse myeloma cells. For example, the MPC 11 $IgG_{2b}$ producing cell line devotes 20-25% of its protein synthetic activity to the production of H and L chains. In absolute terms, these cells are synthesizing 3.6 pg of immunoglobulin/cell/hr or approximately a million H and L chain molecules/cell/min. (21). Since there are a limited number of constant region genes in each cell (see elsewhere in this volume) and probably only one constant region gene is being expressed, this amplification of H and L chain synthesis is not due to gene reiteration. Although the H and L chain genes are not linked (9), relatively equal numbers of the two chains are synthesized. For example, when 18 mouse myelomas were examined, 13 synthesized a significant excess of L chains while 5 produced exactly equimolar amounts of H and L chains (4,22). A heterogeneous population of normal lymph node cells synthesized 1.6 times as many L chains as heavy chains. Since cloned populations of myeloma cells produce the same excess of L chains as the whole population it is unlikely

that the excess L chains are produced by cells that are not synthesizing H chains. In fact we have ruled out this possibility by directly measuring the number of cells producing only L chains in the culture (21).

TABLE III

Types of variants of IgG producing mouse myeloma cells

| | inducing agent | number P3 | number MPC-11 |
|---|---|---|---|
| 1. Synthesis variants | | | |
| a. loss of heavy chain (L chain producer) | spont., NTG, ICR 191 | hundreds | |
| b. loss of light chain (H chain producer) | spont., NTG, ICR 191 | 11 | 0 |
| c. loss of heavy & light chain (non producer) | spont., NTG, ICR 191 | 30+ | |
| d. selective decrease of heavy or light chains | spont. | 1 | 1 |
| 2. Primary sequence variants | | | |
| a. short chain | ICR 191, Melphalan | | 12 |
| | spont. | | 1 |
| b. change in subclass | ICR 191, Melphalan | | 5 |
| 3. Assembly and secretion variants | | | |
| a. secondary to 1 and 2 | | | |
| 1. decreased rates of assembly | | | |
| 2. blocks in assembly | | | |
| 3. blocks in secretion | | | |
| 4. changes in carbohydrate | | | |

These findings raise the question of whether the synthesis of either chain depends upon the synthesis of the other chain. In the P3 cell line this is certainly not true since we have been able to isolate many variants which have lost the ability to synthesize either H or L chains (Table 3) (12 23). Those P3 clones that have lost the ability to synthesize H chains continued to produce approximately the same amount of L chains as the parent. When initially isolated, the H chain producing variants also continued to synthesize the same amount of H chain as the parental cells. However, within weeks synthesis decreased from approximately 5% of th cell protein to 0.5% and then was maintained at that level (23). The H chains synthesized in the absence of L chains were assembled into H chain dimers but were neither degraded nor secreted (23). We have been unable to obtain H chain pro ducing variants from the MPC 11 cell line but the L chain producing variants also continue to synthesize the same amou of L chains as the parental cell lines (Table 3) (21). In fact, variants which synthesize complete H chains but no L chains have not been observed in human myelomas and have so far been reported only in the P3 (21) and MOPC-460 mouse myelomas (24). It is possible that such variants occur frequently but that in most cells the synthesis and intracellula accumulation of large amounts of relatively insoluble H chains is lethal. In the P3 cells, the intracellular accumulation of free H chains appears to result in decreased H chain synthesis, perhaps through a translational mechanism such as that suggested by Stevens and Williamson (25) or mor likely through selection. Those human cells which synthesize only H chains always produce fragments (26) which are presumably more soluble than the intact H chain.

It is of interest that the C1 cell line, which synthesizes exactly equimolar amounts of H and L chains, does not appear to convert to either H or L chain producers but rather simultaneously loses the ability to produce both chain (12). It is possible that in this cell line there is either a linkage of the genes or a very tight coordination of H and L chain synthesis (27).

Those variants that have lost the ability to produce one or both chains provide an additional insight. In the H plus L chain producing parent the percentage of membrane associated ribosomes corresponds to the percentage of immunoglobulin produced. In those few variants studied so far, the loss of immunoglobulin production is not associated with a corresponding loss of membrane associated ribosomes. This

suggests that the continued synthesis of secreted protein is not necessary for the maintenance of an extensive rough endoplasmic reticulum (28).

Our preliminary conclusion from these studies is that the synthesis of either immunoglobulin chain is not dependent on the continued synthesis of the other chain. In the case of H chains, L chain synthesis may be required if H chain synthesis is not reduced by some translational or transcriptional control, but L chain synthesis is probably a secondary requirement to maintain cell viability. The regulation of the amount of membrane associate ribosomes may be separable from the synthesis of secreted proteins.

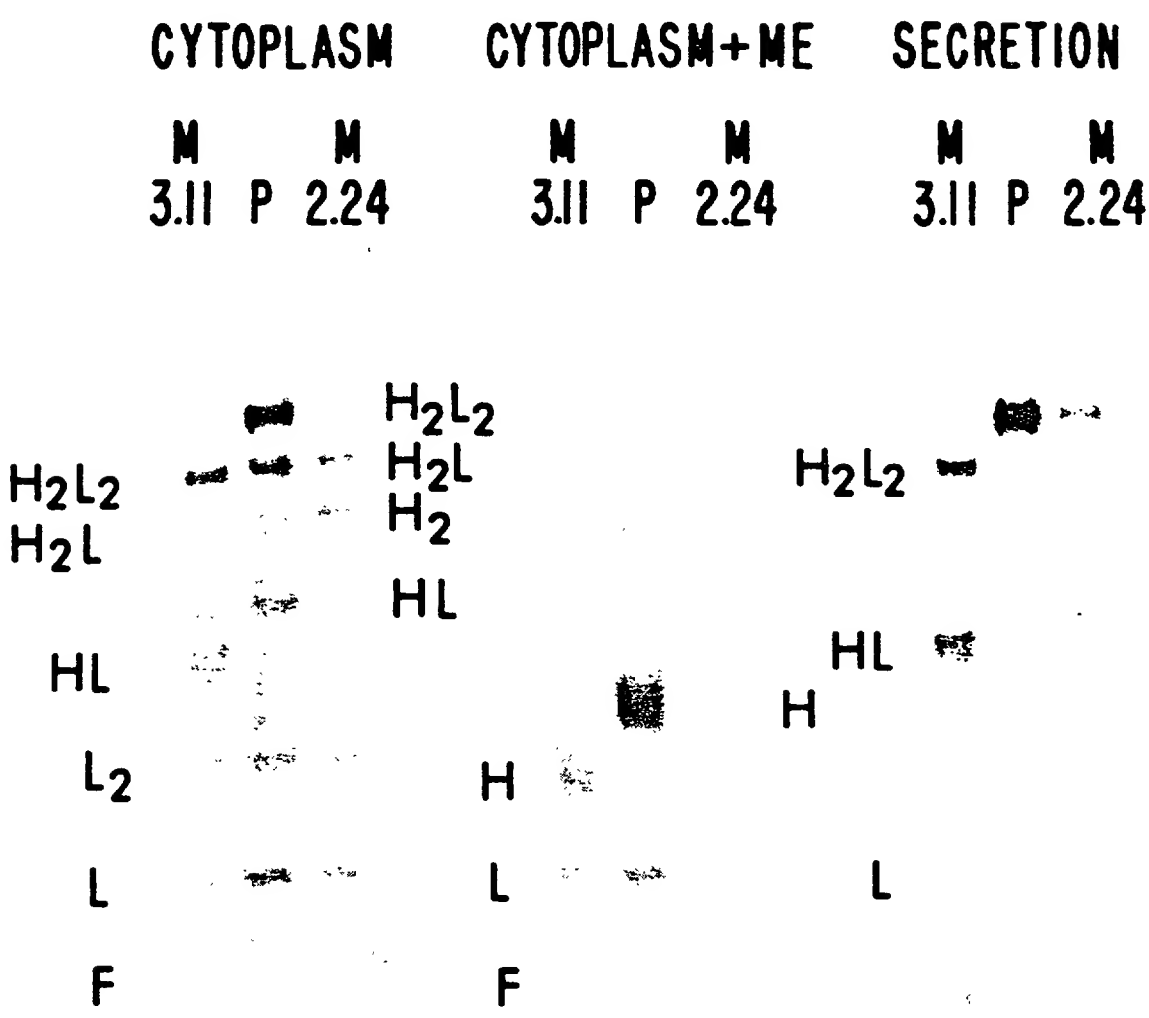
## ASSEMBLY, GLYCOSYLATION AND SECRETION

By comparing a number of mouse myeloma tumors synthesizing the different subclasses of IgG, we were able to show that different subclasses had different predominant covalent and non-covalent pathways of assembly (29,30). Since the L chains of all of these tumors were of the kappa subclass but the H chains differed in their constant regions, it was concluded that the amino acid sequence of the H chains determined the pathway of assembly (29,30). Within each subclass, different tumors showed different kinetics of assembly. These variations could have been due to different variable regions producing differences in the complementarity of the H and L chains or to some property of the cell. Through careful examination of the primary sequence variants we have isolated, it should ultimately be possible to obtain a clearer understanding of the structural features that determine the pathway and kinetics of assembly. The potential of this approach is illustrated in part by Figure 3.

Here we have incubated cells from the parent (P), a short H chain mutant (M3.11) and a 2a positive mutant (M2.24) with radioactive amino acids for 10 min. and then examined the labelled intracellular immunoglobulins by specifically precipitating them from the cytoplasmic lysate of the cell and analyzing them on SDS containing acrylamide gels. Since the SDS does not disrupt the disulfide bonds and the molecules separate based on their size, we can examine the intermediates in the assembly process and estimate the kinetics of their polymerization (31). Following the 10 min. labelling period, the incorporation of radioactive precursors was stopped by "chasing" with a large excess of unlabelled amino acids and the medium was examined for secreted material 3 hrs

later. Since its H chains are smaller than those of the parent (middle panel Figure 3), all of the molecules of M3.11 are also smaller.

Figure 3




The other major differences are: 1) M3.11 does not form $H_2$, i.e. inter-H chain disulfide bonds are not formed prior to the inter H-L chain disulfide bonds; 2) large amounts of HL are secreted providing additional evidence for a block in the formation of the inter-H chain disulfide bonds; and 3) there is a double band for both HL and H chains. This double band is due to the non-glycosylation of a significant percentage of the M3.11 H chains (32). Since $H_2L_2$ and HL both contain non-glycosylated H chains, the carbohydrate moiety on the H chains does not seem necessary for the assembly of this mutant H chain. Furthermore, since the non-glycosylated HL

molecules are secreted with the same kinetics as the glycosylated ones, carbohydrate is not necessary for secretion of H chain containing molecules (32).

M2.24, the 2a positive variant, has a normal sized H chain but differs greatly in sequence from the parental H chain (Table 2). HL, which is the distinguishing precursor in the assembly of the parental $IgG_{2b}$ protein (29,30), is not a precursor of the M2.24 molecule. This is true of normal $IgG_{2a}$ proteins (29,30)confirming that the sequence of the H chain is crucial in specifying the pathway of assembly. In addition, after 10 minutes of labelling, less $H_2L_2$ has been formed in the M2.24 variant than in the parent (17). Since we now have a number of short chain and 2a variants (Table 4) each of which differs from the others in its pattern and kinetics of assembly, glycosylation, and secretion, it should be possible to study the subtleties of these processes in great detail.

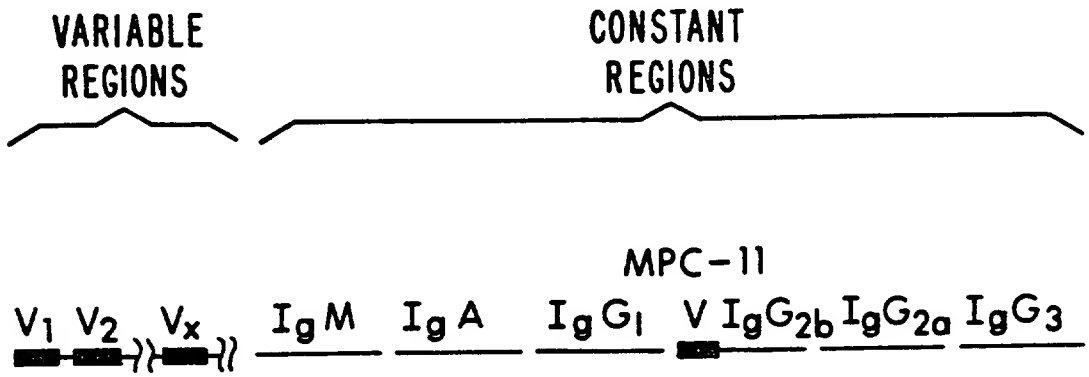
## GENETIC CONTROL OF IMMUNOGLOBULIN PRODUCTION

As already noted, the most provocative finding in all of these studies is the unusually high rate at which variants occur and the increase in this frequency produced by certain mutagenizing agents. Those variants which have lost the ability to synthesize one or the other polypeptide chains could have arisen through chromosome loss, through some epigenetic change similar to those responsible for differentiation, or through true mutation resulting in the changes in the DNA sequence of structural or regulatory genes. These possibilities are being investigated by analysis of somatic cell hybrids in an attempt to complement these defects. In contrast the H chain primary sequence variants have the characteristics of true mutants: they arise through mutagenesis, are stable, and produce a gene product with a change in its amino acid sequence.

A detailed consideration of these primary sequence mutants should provide some insight into the structure and control of the immunoglobulin genes. Since the short chain mutations may be C-terminal and are produced with frame-shift agents (33), the most likely explanation is that a frame shift mutation has occurred in the Fc region with the production of a nonsense mutation and the resulting premature termination of the polypeptide chain. Needless to say, other possibilities such as a deletion in the C-terminal portion of molecule will have to be ruled out by detailed structural analysis.

The mutants that have switched to producing a 2a positive H chain are most easily explained by considering a shematic presentation of the part of the genome containing the H chain constant region genes (Figure 4).

Figure 4




This drawing is based on Gally and Edelman's conception of the genome (34). Of course the order of the constant region genes is not known nor is it clear whether other genes or spacer sequences are interposed. Gally and Edelman (34) have suggested that one of the many available variable region genes is translocated from a neighboring region of the genome onto a constant region gene which in the case of MPC 11 codes for the $IgG_{2b}$ subclass. Assuming the correctness of this thesis and using precedents from microbial genetics, there are three mechanisms which could explain the 2a positive mutants. First, the MPC 11 variable region gene could be "translocated" from the 2b to the 2a constant region gene. This would predict that all of the 2a positive mutants would have identical sequences since they would all reflect the same MPC 11 variable and $IgG_{2a}$ constant region. Since no two of these variants are the same by peptide analysis, this mechanism is unlikely.

The second possibility is that a deletion has occurred between the 2b and 2a constant region genes with the loss of termination and a read-through into the adjoining 2a gene (35). The size of the deletion might vary yielding different size mutant gene products (i.e. the 75,000 dalton protein Table 2). This mechanism predicts polarity. For example, if the scheme presented above were correct, a 2b myeloma could acquire part of a 2a gene, or less frequently part of a $IgG_3$ gene but should never acquire parts of any of the genes

to its left in Figure 4. If one starts with a $IgG_{2a}$ myeloma such as LPC-1, where the LPC-1 variable region is associated with the 2a constant region gene, it should never convert to the 2b subclass but only to the $IgG_3$ subclass etc. The polarity of such subclass switches can be tested and if deletions and read-throughs are occurring they would allow us to map the constant region genome.

The third and probably the most likely mechanism would require mismatching of DNA and crossing over between homologous tandem genes. This crossing over could be equal or unequal resulting in different size recombinant genes and gene products. This sort of mechanism is thought to give rise to Lepore type hemoglobins (36). Expression of such recombinant gene products would not show polarity but would reflect either the distance between genes or the degree of base homology between the different constant regions. In fact Kunkel and Natvig (37,38,39) and their colleagues have reported a series of "hybrid" human immunoglobulin molecules and suggested that they arose through crossing over. This recombinational mechanism raises other interesting possibilities. Since only one of two possible alleles is expressed (40), crossing over between allelic chromosomes could lead to non-expression and explain the high incidence of variants which have lost the ability to synthesize H chains. Crossing over between sister chromatids could lead to the 2a type variants described.

There are of course other possible mechanisms to explain the 2b to 2a switch including the turning on of normally unexpressed constant region genes (41). These and other possibilities can only be resolved by the isolation and detailed characterization of a large number of mutants.

## CONCLUSION

A method for isolating mouse myeloma cells which have undergone mutations in immunoglobulin production has been developed. A variety of mutants have been isolated, their frequency determined, and some of their biochemical properties described. The detailed characterization of such mutants should provide a deeper understanding of the synthesis, assembly, and secretion of immunoglobulin. Examples of the preliminary steps toward such insights are presented here and will also be described by Milstein in the following chapter. Further studies should also allow us to determine whether the high mutation rates observed are due to one of

the unusual aspects of the genetic control of immunoglobuli and provide information on the organization of the immunoglobulin genes.

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## DISCUSSION

G. KOCH: You mentioned transcriptional control and processing as a means of synthesizing immunoglobins up to a level of 20% of the total protein synthesis. I want to propose an additional way involving control at the translation level. Dr. Nuss in our laboratory, has shown that modification of the growth medium increases the amount of immune protein synthesized up to 90% of the total protein and significantly changes the ratio of L-chains and H-chains produced. This indicates that the mRNA for immunoglobins is translated under conditions which block translation of host mRNA.

M.D. SCHARFF: That is what I was alluding to when I referred to the papers by Stevens and Williamson. They never reported 90% of the protein was immunoglobin, but they showed you could change the ratio of heavy and light chains being made, that you could selectively decrease heavy chains synthesis and that you could get changes in immunoglobin synthesis and they ascribed this to a translational control. However, to say that there is a translational control is not to specify the mechanism by which the immunoglobin synthesis is amplified. My reading of the amount of messenger present is

that in a myeloma tumor or cell line there is enough messenger to code for the amount of immunoglobin that is being made. I think that what you say is very interesting, that one can exert translational modulation superimposed on other controls, but the initial amplification still must result in the synthesis of enough messenger to make the immunoglobin and I guess that has to be either transcription or some sort of processing.

G. KOCH: Of course, I agree with that. I don't want to say that you don't have transcriptional control. I would suggest, however, that the mRNAs for immunoglobin have a higher affinity for ribosomes than do other mRNAs and that this also provides amplification for the synthesis of immunoglobins.

S. RAAM: You mentioned that you have worked with IgM secreting murine tumor cells. Did you come across any mutants with regard to J-chain biosynthesis and assembly?

M.D. SCHARFF: The answer is that we have not looked, because we have not been able to make antiserum against J-chain. However, it has been reported by Kaji and Parkhouse that IgG producing cells, as well as IgM and IgA producing myelomas, synthesise the J-chain found in the polymers of IgA and IgM.

R. SCHULMAN: Have you looked for reversion of short chain mutants by base analogs?

M.D. SCHARFF: I should have said something about that. We have not found a revertant anywhere. We tried to revert the original mutants with the mutagenizing agent that we used to produce them, and also with other agents, but in no case did we obtain reversion. There is a problem as to what one calls a revertant. We have found phenotypic changes. For instance, a variant that does not secrete and makes heavy chain of $50 \times 10^3$ can begin to secrete spontaneously. After it starts secreting, however, it makes heavy chains of $39 \times 10^3$, which for some reason or other, the cell is able to secrete. We have not found a legitimate structural revertant that has achieved a normal size chain.

C. BELL: Do you know whether the different synthesising capacities of the parental type myeloma cell synthesising $H_2L_2$ and the subsequent variants synthesising or secreting HL or $H_2$ are due to different mRNA's or to some other control mechanism preventing translation of mRNA in these variants?

M. D. SCHARFF: The only thing that we know is that in the cells that have lost the ability to make heavy and light chains Kuehl and Leder have failed to detect light chain messenger that has the same size as the parent. It could not be detected by either hybridization or translation. We know nothing about heavy chain messages.

E.A. KABAT: Have you found any evidence of V-region mutants?

M.D. SCHARFF: When we saw the high mutation rates we hoped that we were going to prove the somatic generation of antibody diversity. However, all the variants that I have described to you are in the constant region. By using antigens in the overlay to look at antigen binding myelomas, we have found some myelomas which secrete immunoglobins normally but no longer bind the antigen. One would guess that these have defects in the variable region. If we are finding variable region mutants they are not arising at much higher rates than constant region ones. I have one qualification. I have told you mainly about deletion mutations. We have not found any single amino acids substitutions so far. Although we have looked with the correct mutagenizing agents, our assay for finding them might well miss them. I think that is an important qualification because it could be that there are variable region changes occurring which we are not picking up either by isoelectric focusing or by defects in the assembly or secretion.

Exhibit C

# THE SYNTHESIS, ASSEMBLY, AND SECRETION OF IMMUNOGLOBULIN: A BIOCHEMICAL AND GENETIC APPROACH

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# THE SYNTHESIS, ASSEMBLY, AND SECRETION OF IMMUNOGLOBULIN: A BIOCHEMICAL AND GENETIC APPROACH*,†

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## I. Introduction

Attempts to investigate the regulation of protein synthesis in higher organisms are frequently frustrated by the complexity of the systems which must be used. One tactic for dealing with this is to study homogeneous populations of cells which are synthesizing large amounts of a single protein. Cells obtained from mouse myeloma tumors provide such a system: they are highly differentiated plasma cells which synthesize and secrete large amounts of a single immunoglobulin; they can be adapted to continuous culture and cloned in soft agar; and most important, the immunoglobulin molecules which they produce have been well characterized both physically and chemically (Potter, 1972). In addition, the structure of the immunoglobulin molecule makes the myeloma system an excellent model for studying the synthesis and assembly of multichained proteins. Finally, many myeloma tumors making each of the subclasses of mouse immunoglobulin are available.

Initially workers in a variety of laboratories utilized tumors to study the synthesis, assembly, and secretion of mouse immunoglobulins and to compare these processes with those in normal and malignant cells from other species (Scharff and Laskov, 1970; Williamson, 1971; Buxbaum, 1973). More recently cultured cells have been used to study the genetic control and molecular biology

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of immunoglobulin production. In the sections that follow, I will briefly summarize our contributions to both these areas.

## II. Synthesis, Assembly, and Secretion of Mouse Immunoglobulin

These processes are schematically presented in Fig. 1. After the immunoglobulin messenger is transcribed in the nucleus and transported into the cytoplasm, it becomes associated with membrane-associated ribosomes to form the rough endoplasmic reticulum where the messenger is translated in the heavy and light immunoglobulin polypeptide chains. While there is evidence that immunoglobulin may also be synthesized on free polyribosomes (Sherr and Uhr, 1970; Lisowska-Bernstein *et al.*, 1971; Zauderer, 1973), it is not clear whether this is due to some sort of artifact or, if true, is quantitatively significant. In any case, most of the newly synthesized heavy and light chains rapidly appear within the cisternae of the endoplasmic reticulum, are assembled into covalently linked molecules, and acquire carbohydrate (Uhr, 1970; Sutherland *et al.*, 1972). Most of the immunoglobulin molecules are then secreted relatively rapidly. At the same time a small percentage of the newly synthesized immunoglobulin is inserted into the plasma membrane and remains there for many hours (Uhr, 1970; Knopf, 1973). A detailed understanding of

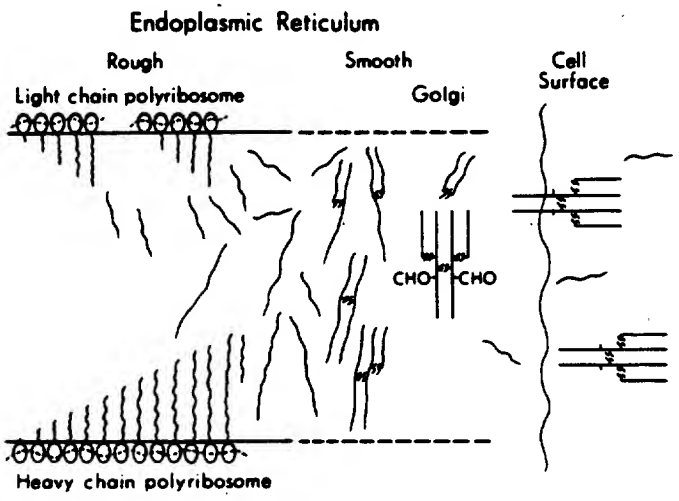


FIG. 1. Schematic representation of the synthesis, assembly, and secretion of mouse immunoglobin. —ss—, disulfide bond.

these events would provide significant insight into the more general problems of the regulation of protein synthesis and the assembly of complex proteins, the mechanism of secretion, and the metabolism of membrane glycoproteins in animal cells.

In order to obtain a better understanding of these processes, we started out by asking a number of simple and specific questions: (1) Is the synthesis of heavy and light chains coordinated? (2) How quickly are the newly synthesized heavy and light chains asembled, and how complete is the assembly process? (3) Are there specific pathways of assembly? (4) What are the kinetics and requirements for secretion?

### *A. Coordination of Heavy and Light Chain Synthesis*

We examined a number of mouse myeloma tumors to determine whether heavy and light chains were made in exactly equimolar amounts, whether one chain was always made in excess, or whether coordination was completely relaxed with some tumors synthesizing excess light chains, some excess heavy chains, and some equal amounts of both chains. This question was of special interest because the immunoglobulin polypeptide chains are made in larger amounts than most cellular proteins and their synthesis must therefore be amplified by gene dosage, increased transcription, translation, or more efficient messenger utilization. Since the heavy and light chain genes are not linked and are translated on separate messengers (cf. Scharff and Laskov, 1970), any amplification process would have to work independently on both chains. The synthesis of IgG was therefore examined in normal lymph node cells from hyperimmunized mice and in 18 different IgG-producing mouse myeloma tumors and cell lines (Baumal and Scharff, 1973a). The heterogeneous population of normal cells secreted 1.6 times as many light chains as heavy chains (Table I). Five of the 18 myeloma tumors synthesized equimolar amounts of heavy and light chains while the other 13 tumors and cell lines produced between a 1.2- and 4.2-fold excess of light chains. A few of the myeloma tumors which produced excess light chains degraded them intracellularly, but most secreted all the excess light chains they synthesized (Table I) (Baumal and Scharff, 1973a). A similar study of 16 human myeloma patients revealed

TABLE I

SYNTHESIS OF IgG IN MOUSE CELLS[a,b]

| | Percent cell protein as IgG | Percent IgG as free L chain | L/H molar ratio: Intracellular 3 Min | 9 Min | 15 Min | Secretion 3 Hours |
|---|---|---|---|---|---|---|
| Immune lymphoid cells | 30 | 12 | 1.7 | 1.5 | 1.5 | 1.6 |
| Myeloma tumors and cell lines (18) | 29 | 11 | 2.2 | 2.0 | 1.9 | 1.8 |
| Synthesis of excess L chains | | | | | | |
| a. Without degradation (11) | | | | | | |
| MOPC-245 | 43 | 7 | 1.6 | 1.7 | 1.6 | 1.5 |
| MOPC-282 | 21 | 16 | 2.8 | 2.8 | 2.7 | 2.8 |
| MPC-11 | 25 | 32 | 3.5 | 3.4 | 3.6 | 3.6 |
| b. With degradation (2) | | | | | | |
| MOPC-21 | 30 | 0 | 3.4 | 2.2 | 1.7 | 1.0 |
| Balanced synthesis (5) | | | | | | |
| Adj. PC-5 | 29 | 0 | 0.9 | 1.0 | 1.1 | 1.0 |

[a]The details of these experiments are described in Baumal *et al.* (1971) and Baumal and Scharff (1973a). Briefly, single cell suspension from normal lymphoid cells and from each of 18 myelomas were incubated with $^{14}$C-labeled amino acids for 30 minutes, and the percentage of the newly synthesized proteins which was specifically precipitable as immunoglobulin was determined. The immunologic precipitates were analyzed on SDS-containing acrylamide gels to determine the percent of immunoglobulins which were free light chains or light chain dimers.

[b]Degradation of heavy (H) or light (L) chains was examined by incubating cells with radioactive amino acids for 5 minutes, chasing with a large excess of unlabeled amino acids, and then immunologically precipitating the intracellular and secreted immunoglobulins at various times after the chase. The specific precipitates were reduced and alkylated and the ratio of heavy and light chains determined.

4 patients with apparently balanced synthesis and 12 producing excess light chains (Zolla *et al.*, 1970).

Since cloned populations of myeloma cells produced the same excess of light chains as the tumor as a whole (Baumal and Scharff, 1973a), it is unlikely that the synthesis of excess light chains was the result of a few cells that were producing only light chains. In fact, we ruled out this possibility in at least one

instance where the number of light chain-producing cells was measured directly (Laskov and Scharff, 1974).

In summary, heavy and light chain synthesis was stringently coordinated in some myelomas and loosely controlled in others. However, in no case were excess heavy chains synthesized. Stevens and Williamson (1973) have suggested that heavy chain synthesis may be controlled translationally. The synthesis of excess light chains is obviously not necessary since some tumors did produce equimolar amounts of the two chains. However, it is possible that it is of some advantage to the cell to have a small pool of free light chains to bind to the relatively insoluble heavy chains either as they are completed on the polyribosome or just after they are released.

### *B. Kinetics and Completeness of Assembly*

Much is known about the *in vitro* refolding of polypeptide chains and the *in vitro* reassembly of dissociated heavy and light chains has been studied extensively (Dorrington and Tanford, 1970). However, less is known about how complex proteins are assembled within the intact cell. Does the assembly process begin while the polypeptides are still on the polyribosome, or are the chains first released from the ribosome and then find each other? How efficient is the assembly process, and does it take seconds, minutes, or hours? How long after the chains become associated do they form disulfide bonds? Are there specific pathways of assembly, and if so what determines the pathway to be used?

The covalent structure of the immunoglobulin molecule with its different sized chains makes it an especially useful model protein for answering these questions. Pulse-chase experiments were therefore carried out on a number of IgG-producing mouse myeloma tumors and on normal lymph node cells (Laskov *et al.*, 1971; Baumal *et al.*, 1971). After a 1–3-minute pulse, it took 2 minutes for all the nascent heavy and light chains to be released from the ribosomes (Shapiro *et al.*, 1966; Laskov *et al.*, 1971). Any assembly which occurred after that time must therefore have taken place between already released polypeptide chains. In fact, in most mouse myelomas, relatively little noncovalent or covalent

assembly occurs during the first few minutes after release of the heavy and light chains from the ribosome. Seven minutes after the chase, only 37% of the newly synthesized chains had been fully assembled into $H_2L_2$ IgG molecules (Table II) (Baumal *et al.*, 1971). Some tumors such as MOPC-31 were very slow while in others assembly was more rapid. When compared to the average behavior of the myeloma tumors studied, assembly occurs more rapidly in normal lymph node cells (Table II). However, in both myelomas and normal lymph node cells, assembly was not complete, and intermediates and free light chains were secreted as such (Table II) (Baumal *et al.*, 1971).

Polymerization of the $H_2L_2$ monomer into higher polymers occurs in the assembly of IgA and IgM. In most, but not all, of the mouse myelomas studied this step occurs close to the time of secretion (Parkhouse and Askonas, 1969, 1971; Bargellesi *et al.*, 1972; Buxbaum and Scharff, 1973). Buxbaum *et al.* (1971) have studied this process in humans and found that, in contrast to mouse myeloma cells, the cells of patients with Waldenström macroglobulinemia usually contain intracellular polymers.

### C. *Pathways of Assembly*

The assembly of immunogloublin molecules from their constituent heavy and light chains could theoretically occur via a

TABLE II

KINETICS AND COMPLETENESS OF ASSEMBLY OF MOUSE IgG

| Source | Percent assembled intracellularly | | Percent assembled into $H_2L_2$ in secretion |
|---|---|---|---|
| | 7 Min | 18 Min | |
| Immune lymph nodes | 22 | 53 | 75 |
| Average 16 myelomas | 37 | 59 | 76 |
| MOPC-31 | 10 | 35 | 91 |
| MOPC-282 | 57 | 77 | 80 |
| MOPC-141 | 33 | 64 | 70 |
| MPC-11 (tumor) | 26 | 36 | 36 |

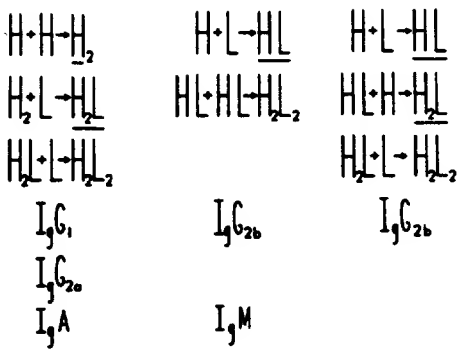


FIG. 2. Preferred pathways of interchain disulfide formation. Intermediates in the assembly process are underlined. H, heavy chain; L, light chain.

number of pathways in which different intermediates are formed. Based on the arrangement of the intramolecular disulfide bonds (see Fig. 1), there are three likely sequences of interchain disulfide formation (Fig. 2). The pathway used can be identified by the intermediates (underlined in Fig. 2) in the assembly process. Studies of many mouse myeloma tumors, a more limited sample of human myelomas, and normal mouse and rabbit lymph node cells have revealed that there are preferred pathways of assembly (cf. Scharff and Laskov, 1970; Baumal *et al.*, 1971). However, in all the tumors studied, a small percentage of the heavy and light chains were assembled through the alternative pathway. The situation in the BALB/c mouse is illustrated in the Fig. 2 where the $IgG_1$ and $IgG_{2a}$ subclasses and IgA assemble predominantly through the $H_2$ to $H_2L$ (left side of Fig. 2) pathway while $IgG_{2b}$ and IgM assemble primarily through HL half-molecules. These pathways of assembly reflect not only the sequence of formation of the intermolecular disulfide bonds, but also the order of noncovalent associations of the polypeptide chains (Baumal *et al.*, 1971). The structure of the heavy chain appears to determine the pathway of assembly since almost all the mouse tumors studied synthesized kappa light chains.

### *D. Secretion*

After the immunoglobulin polypeptides are released into the cisternae of the rough endoplasmic reticulum, they progress through the smooth endoplasmic reticulum to the Golgi apparatus

and then by unknown mechanisms (Uhr, 1970) to the cell surface.

In both normal and malignant plasma cells, there is at least a 20-minute lag between the time of synthesis and the time of secretion. In normal mouse lymph node cells, most of the molecules which are synthesized at a given time are secreted between 40 and 80 minutes later (Baumal and Scharff, 1973b). This confirms the earlier studies by Helmreich *et al.* (1962), who showed that rabbit immunoglobulin was secreted by "seniority" indicating that the newly synthesized proteins progressed through the cell in a tightly ordered progression. In contrast, mouse and human myeloma cells secrete their immunoglobulins in a more random way with the newly synthesized molecules appearing outside the cell any time between 20 and 150 minutes after they are synthesized (Scharff and Laskov, 1970; Baumal and Scharff, 1973b).

## III. Somatic Cell Genetics of Immunoglobulin Production

The results described above taken together with a variety of studies from other laboratories (cf. Scharff and Laskov, 1970; Williamson, 1971; Buxbaum, 1973) provide a general description of the synthesis, assembly, and secretion of mouse immunoglobulin by myeloma and, to some extent, by normal lymph node cells. They suggest that a variety of transcriptional, translational, and posttranslational regulatory mechanisms play a role in the production of immunoglobulin by plasma cells. However, these largely descriptive studies do not reveal the detailed mechanisms responsible for these events and raise many additional questions. How do the immunoglobulin messengers segregate to the membrane associated ribosomes? What determines whether an immunoglobulin molecule will become membrane associated or be secreted? Does the carbohydrate play any role inside the cell? Do any specific cell functions facilitate the assembly process?

It is clear that attempts to answer these questions, and to understand the molecular mechanisms responsible for each of the steps in the production of immunoglobulin, would be greatly facilitated if mutants blocked at each of these steps could be isolated. It

possible that such mutants would also provide insight into the genetic and biochemical mechanisms responsible for the generation of antibody diversity, the suspected translocation of variable and constant regions, or allelic exclusion.

### *A. The Isolation of Variants*

In order to find mouse myeloma cells which had undergone a change in immunoglobulin production, the MPC-11 tumor, which produces $IgG_{2b}$ immunoglobulin, was adapted to continuous culture (Laskov and Scharff, 1970). The cultured cells continued to produce large amounts of the same immunoglobulin synthesized by the tumor. The cells were cloned at high efficiencies in soft agar using primary, secondary, or tertiary rat embryo fibroblasts as feeder layers. Three to four days later, the colonies were overlayered with agar containing antibody against one or both of the immunoglobulin polypeptide chains (Coffino *et al.*, 1972). When the dishes were examined 2–3 days later under low power with an inverted microscope, most of the colonies were surrounded and obscured by antigen–antibody precipitates. However, an occasional clone was "unstained." If the antiserum used to overlay the clones was specific for heavy chains, then the unstained clones either: (1) had lost the ability to synthesize heavy chains; (2) continued to synthesize heavy chains but were unable to secrete them; or (3) were secreting heavy chains which no longer contained the antigenic determinants present in the parental heavy chain (Coffino *et al.*, 1972). These various alternatives were examined by recovering the presumptive variants from the agar, growing them to mass culture, and then examining the intracellular and secreted immunoglobulin using agar diffusion. Detailed studies of the variants were carried out by incubating the cells with radioactive precursors, immunologically precipitating the intracellular and secreted immunoglobulins, and then dissolving the specific precipitates with sodium dodecyl sulfate (SDS) and analyzing the radioactive material on SDS-containing acrylamide gels (Coffino and Scharff, 1971).

When a freshly isolated MPC-11 clone was examined with antibody against heavy chains, 0.5% of the clones were unstained.

Experiments with 3 other IgG, 2 IGA- and an IgM-producing cell line, revealed a similar surprisingly high incidence of clones which no longer stained with antibody directed against the parental heavy chains. A large number of spontaneous MPC-11 variants which had lost the ability to react with antibody against heavy chains have been recovered and studied in detail. Intracellular heavy chains have not been detected in any of these variants, but all continued to synthesize and secrete light chains in approximately the same amounts as the parental heavy plus light chain-producing clone (Coffino and Scharff, 1971; Kuehl and Scharff, 1974; Laskov and Scharff, 1974). When the same approach was used with antibody against light chains, spontaneously occurring nonproducing variants were also isolated (Coffino and Scharff, 1971; Kuehl and Scharff, 1974).

Because of the surprisingly high incidence of variants, fluctuation analyses were carried out on three IgG-producing cell lines. Statistical analysis revealed that the variants arose spontaneously and were not induced by the assay. The parental MPC-11 heavy plus light chain-producing cells converted to light chain producers at a rate of $1.1 \times 10^{-3}$/cell per generation. No revertants were found (Fig. 3) (Coffino and Scharff, 1971; Baumal *et al.*, 1973). The P3 and C1 cell lines lost the ability to produce heavy chains at a similarly high rate (Fig. 3) (Baumal *et al.*, 1973). However, the C1 cell line converted directly to nonproducers. C1 also differs from the other cell lines studied in that it arose as a spontaneous

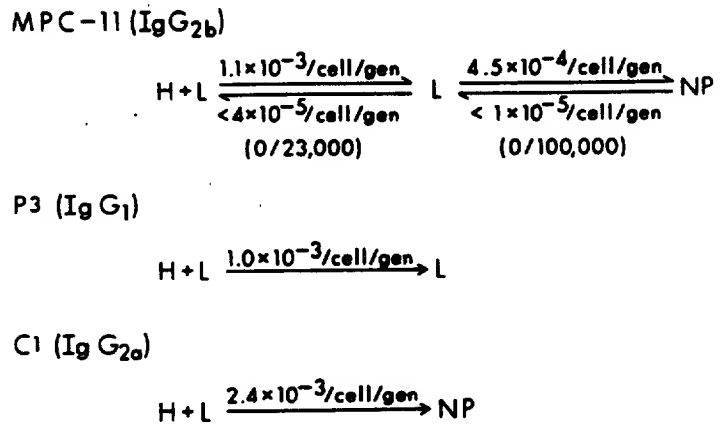


FIG. 3. "Mutation" rates for immunoglobin production for cell lines MPC-11, P3, and C1. H, heavy chain; L, light chain; gen, generation; NP, nonproducer.

tumor in a C3H mouse and makes exactly equimolar amounts of heavy and light chains whereas P3 and MPC-11 are of BALB/c origin and make excess light chains.

Because the "mutation" rate for immunoglobulin production was so high, the MPC-11 cell line was examined for spontaneous occurring variants resistant to the drugs bromodeoxyuridine, thioguanine, puromycin, and deoxyglucose (Baumal *et al.*, 1973). The incidence of such drug-resistant variants was less than $10^{-6}$, which is comparable to rates reported in other cell lines.

These findings suggest that the myeloma cells are not hypermutable for all traits and that the high incidence of immunoglobulin variants is in some way specifically related to the genetic control of immunoglobulin production.

A few of the spontaneously occurring light chain and nonproducing variants of MPC-11 have been examined in more detail. These contained approximately the same heteroploid number of chromosomes, the same virus particles and surface antigens, and the same number of membrane-associated ribosomes as the parent. The size of the membrane-associated polyribosomes and their ability to incorporate radioactive amino acids was also the same as that of the parent, which devotes 20% of its protein synthetic activity to heavy and light chain synthesis (Kuehl and Scharff, 1974). This finding was very surprising, since we had assumed that the cell would lose its rough endoplasmic reticulum when it stopped making secreted proteins. Studies now in progress indicate that the loss of ability to synthesize complete light chains is due to a lack of light chain messenger (Kuehl *et al.*, 1975).

### *B. Mutagenesis*

The above studies demonstrated the usefulness of the techniques we had developed. The high incidence of variants was provocative, but in the absence of either revertants or mutant gene products it was difficult to investigate the genetic basis for the high incidence of variants. We therefore began to study the effect of some representative mutagenizing agents with the hope that (1) some of these agents would be more effective than others and thus provide some insight into the genetic basis for the ob-

served mutation rates; and (2) we could obtain some primary sequence variants which, when chemically characterized, would allow us to deduce the types of changes which were occurring in the DNA.

Ethylmethane sulfonate, an alkylating agent, did not result in a significant increase in the number of variants, and nitrosoguanidine caused only a 2- to 3-fold increase in unstained colonies (Table III) (Baumal *et al.*, 1973). However, ICR-191, an acridine half-mustard which causes frameshift mutations in microorganisms, caused as many as 6% of the surviving cells to become variants (Table III). Melphalan, which is a phenylalanine mustard used to treat human myelomas, was also a very effective mutagen (Table III) (Preud'homme *et al.*, 1973).

Most of the mutagen-induced variants resembled the sponta-

TABLE III

EFFECT OF MUTAGENS ON THE CONVERSION OF HEAVY (H) AND LIGHT (L) TO L CHAIN PRODUCERS [a,b]

| Agent | Mutagen (μg/ml) | Percent cell survival | Incidence of variants | Percent | *P* |
|---|---|---|---|---|---|
| EMS | 0 | 100 | 13/2168 | 0.6 | — |
| | 100 | 45 | 10/1288 | 0.8 | 0.45 |
| NTG | 0.25 | 90 | 46/3828 | 1.2 | 0.002 |
| ICR 191 | 0 | 100 | 18/2104 | 0.86 | — |
| | 1 | 60 | 56/3635 | 1.54 | 0.016 |
| | 2 | 25 | 110/3404 | 3.24 | <0.001 |
| | 4 | <1 | 15/229 | 6.55 | <0.001 |
| Melphalan | 0 | 100 | 17/3777 | 0.45 | — |
| | 0.2 | 32 | 31/2336 | 1.33 | <0.001 |
| | 0.6 | 16 | 55/2961 | 1.86 | <0.001 |
| | 0.8 | 9 | 31/1298 | 2.39 | <0.001 |

[a] The details of these experiments are described in Baumal *et al.* (1973). Cells were grown in the presence of various doses of each mutagen for 24 hours, then grown in the absence of the mutagen for an additional 24 hours. The cells were then cloned in soft agar and overlayered with antibody against heavy chains; the total number of clones and percent of unstained clones were determined. *P* represents the statistical significance of results determined by the $\psi^2$ test.

[b] EMS, ethylmethane sulfonate; NTG, nitrosoguanidine; ICR-191, an acridine half-mustard.

TABLE IV

CHARACTERISTICS OF VARIANTS THAT PRODUCE "DEFECTIVE" HEAVY CHAINS[a]

| Mutagen | Molecular weight of H chain | Idiotype | Fc | $IgG_{2b}$ | $IgG_{2a}$ | Tryptic–chymotryptic peaks in common with parent |
|---|---|---|---|---|---|---|
| 0 | 55,000 | + | + | + | − | |
| Melphalan | 44,000 | + | − | − | − | 27/35 |
| ICR | 50,000 | (N.D.) | − | − | − | 26/30 |
| Melphalan | 55,000 | + | + (spur) | − | + | 18/34 |
| ICR | 75,000 | + | + (spur) | − | + | 18/34 |

[a] Molecular weights were determined on sodium dodecyl sulfate-containing acrylamide gels. Cell lysates were examined with anti-Fc, anti-$IgG_{2b}$, and anti-$IgG_{2a}$ by double diffusion in agar. Peptide analysis was carried out by mixing the parent heavy chain labeled with $^{3}H$ amino acids with the variant heavy chain labeled with $^{14}C$-amino acids, digesting with trypsin and chymotrypsin, and then chromatographing the digest on Dowex 50 columns using a pyridine–acetate buffer system (Laskov and Scharff, 1970). ND, not done.

neously occurring ones in that they had lost the ability to produce heavy chains. However, 30–40% of the variants continued to synthesize heavy chains, which were chemically and serologically different from those produced from the parent (Birshtein *et al.*, 1974).

A number of variants producing "defective" heavy chains have now been isolated, and the characteristics of a few representative ones are summarized in Table IV. Some of the variants synthesized heavy chains which were smaller than those of the parent. These "small chain" variants no longer reacted with antibody to the Fc (C-terminal half of the heavy chain) or subclass ($IgG_{2b}$) specific antigens of the parent. However, the variant immunoglobulin still had the MPC-11 idiotype (Rose Lieberman, personal communication) indicating that at least part of the N-terminal end of the molecule was intact. Analysis of the tryptic-chymotryptic peptides revealed that the small-chain variants lacked 6–8 of the peptides found in the parent (Table IV). Variants

producing 50,000-dalton heavy chains were blocked in the formation of the inter-H chain disulfide bond and in secretion. Variants producing 44,000-dalton heavy chains were only partially blocked in the formation of the inter-H chain disulfide bond and secreted in an almost normal fashion (Birshtein *et al.*, 1974; Weitzman and Scharff, 1975).

The simplest explanation of all these findings is that the "short chain" variants have undergone a deletion somewhere in the C-terminal half of their heavy chains. Perhaps there has been a frameshift mutation with a premature termination. Secher *et al.* (1973) has described two spontaneously occurring variants of the P3 cell line which also have short heavy chains. One lacks the C-terminal end of the molecule, and the other has an internal deletion.

In addition to the short-chained variants, we have isolated a number of clones which produce heavy chains that are the same size or larger than the parental heavy chain (Table IV). These chains differ greatly from the parent by peptide analysis, no longer react with antibody against the $IgG_{2b}$ determinants, and most significantly have acquired the serotype of the $IgG_{2a}$ constant region (Preud'homme *et al.*, 1975). Peptide analysis revealed a number of 2a specific peptides. In addition, the 2a positive variants assemble like $IgG_{2a}$ through $H_2$ and $H_2L$ intermediates rather than through the 2b pathway (Fig. 2). One of these 2a positive variants was much larger than the parental heavy chain (Table IV).

It is possible that these 2a positive variants arose either through crossing over between closely related tandem constant region genes or are the result of a deletion running from the C-terminal end of the 2b constant region to the N-terminal part of the 2a constant region with a "read-through" into the 2a constant region. Similar "hybrid" proteins are sometimes produced by mouse (Warner *et al.*, 1966) and human (Kunkel *et al.*, 1969; Natvig and Kunkel, 1974) myeloma tumors.

The detection and characterization of primary sequence variants is of special interest because there has been some question as to whether variants which arise in cultured cells are "mutants," i.e., whether they represent a change in the sequence of chromosomal DNA (Harris, 1971; Mezgar-Fried, 1972). The variants

summarized in Table IV fit many of the criteria of true mutants: their incidence is increased by mutagenesis, they are usually stable, and, most important, the mutant gene product differs in its primary sequence from the parental heavy chain. Furthermore, it is possible to suggest mechanisms for the generation of these variants which are similar to those which have been observed in microorganism.

However, the most provocative aspect of these findings is the very high rate at which these presumed mutations are generated. Normally, this would suggest some sort of epigenetic phenomenon. For example, the variants could represent the turning on of genes which are not usually expressed. It is possible that the detailed structural analysis of a number of mutant MPC-11 chains which is now being carried out in Dr. Barbara Birshtein's laboratory will make it possible to discriminate between epigenetic and genetic mechanisms.

Based on the characteristics of the variants described above, it is quite possible that the high incidence of variants is related to some unusual property of the biochemical genetics of the immunoglobulin genes. Although the mouse myeloma cells are heteroploid, because of allelic exclusion it is likely that the immunoglobulin genes are functionally haploid. This would of course increase the chance of detecting recessive mutations. In addition, the constant region genes for the different heavy-chain subclasses are presumed to be closely linked and therefore represent a series of homologous tandemly arranged genes which might be prone to mismatching. It has also been suggested that during the immune response a single variable region is translocated from the IgM constant region to the IgG constant region. If there is in fact some mechanism for somatic translocation (Gally and Edelman, 1972), it could also promote the sorts of changes we are observing in the $IgG_{2a}$ positive variants. Finally, there is the possibility that antibody diversity is generated in somatic cells. Unfortunately, the methods we have used to detect variants look at the constant rather than the variable region, so it is not surprising that all of the primary sequence variants detected so far have their defect in the constant region. However, our present evidence does not suggest that we are observing anything related

to the putative somatic generation of antibody diversity. Whatev the mechanism for the high incidence of mutants, we have su ceeded in obtaining a number of variants blocked in one c another step in immunoglobulin production which will allow u to gain additional insights into the synthesis, assembly, secretior and regulation of immunoglobulin production.

## IV. Summary

Mouse myeloma tumors and cell lines have been used to de scribe the synthesis, assembly, and secretion of mouse immuno globulin molecules. Most myeloma tumors and normal lymphoid cells synthesize an excess of light chains, but in some tumors exactly equimolar amounts of heavy and light chains are produced. Assembly into the $H_2L_2$ molecules occurs after release of the polypeptide chains from the ribosome, requires a number of minutes, and is incomplete. Different subclasses of immunoglobulin have different preferred pathways of assembly.

A method has been developed for detecting myeloma cells which have undergone variation in the production of immunoglobulin. Such variant cells arise spontaneously at a very high rate which is increased significantly by mutagenesis. A number of primary sequence mutants have been isolated which are blocked in assembly and secretion. Preliminary structural and serological analysis of the mutant heavy chains indicates that some may have arisen as a result of frame shifts or deletions whereas others could have resulted from unequal and equal crossing over.

Identification and characterization of additional mutants may provide us with some insight into the organization and regulation of the immunoglobulin genes.

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Exhibit D